

(19)



Europäisches Patentamt

European Patent Office

Office européen des brevets



(11)

EP 0 864 648 A1

(12)

EUROPEAN PATENT APPLICATION

published in accordance with Art. 158(3) EPC

(43) Date of publication:

16.09.1998 Bulletin 1998/38

(21) Application number: 97941189.9

(22) Date of filing: 18.09.1997

(51) Int. Cl.⁶: **C12N 15/09**, C12N 15/11,
C12Q 1/02, C12Q 1/25,
G01N 33/53, C07C 50/06,
C07C 50/30, C07C 66/00,
C07D 295/16, A61K 31/12,
A61K 31/165, A61K 31/215

(86) International application number:

PCT/JP97/03303

(87) International publication number:

WO 98/12313 (26.03.1998 Gazette 1998/12)

(84) Designated Contracting States:

AT BE CH DE DK ES FI FR GB GR IE IT LI LU MC
NL PT SE

(30) Priority: 20.09.1996 JP 250697/96

(71) Applicant: SUNTORY LIMITED

Kita-ku, Osaka-shi, Osaka 530 (JP)

(72) Inventors:

• NUNOKAWA, Youichi
Osaka 560 (JP)

• OIKAWA, Shinzo

Nishikyo-ku Kyoto-shi Kyoto 615 (JP)

• TANAKA, Shoji

Hyogo 659 (JP)

(74) Representative:

VOSSIUS & PARTNER

Siebertstrasse 4

81675 München (DE)

(54) **METHOD FOR SCREENING COMPOUNDS REGULATING THE EXPRESSION OF HUMAN-INDUCIBLE NITRIC OXIDE SYNTHASE**

(57) A transformant prepared by constructing a plasmid wherein a human-inducible nitric oxide synthase (hiNOS) structural gene has been substituted by a luciferase structural gene which is a reporter gene while sustaining the functions of the 5'-promoter region and the 3'-untranslated region of the hiNOS gene and then stably transferring the plasmid into an established human cell line. This transformant expresses selectively the reporter gene in the presence of an inducer. By determining the expression dose of the reporter gene owing to this transformant, it is possible to conveniently and sensitively screen compounds seemingly being useful in treating inflammation or sepsis through the inhibition of the expression of hiNOS. By inducing the expression thereof, further, it is possible to conveniently and sensitively screen compounds seemingly being useful as antitumor agents or antiviral agents or in treating re-angiostenosis inhibition.

EP 0 864 648 A1

Description

Technical Field

The present invention relates to a method for screening a compound capable of influencing the expression level of human inducible nitric oxide synthase, more specifically to a method for screening a compound capable of controlling the expression of the enzyme.

Background Art

Since when nitric oxide (NO) was identified as a vasodilation factor, it has been found to be a physiologically active substance that plays an important role in regulating the biological functions. In addition to the above function, it was reported that NO has a platelet aggregation suppressing effect, a neurotransmitter releasing effect, and an effect to cause macrophage to exhibit antitumor and bactericidal activities (Moncada, S. and Higgs, A. (1993) *N. Engl. J. Med.* **329**, 2002-2012).

NO is biologically synthesized by NO synthase (NOS) from L-arginine as a substrate. At present, three isozymes of this enzyme have been confirmed to exist (the brain type, the endothelial type, and the inducible type). Their chromosomal localization is also known (Knowles, R. G. and Moncada, S. (1994) *Biochem. J.* **298**, 249-258).

Among these, the inducible type NOS (iNOS) can be expressed by applying endotoxins or cytokines to such cells as vascular smooth muscle cells (VSMC), hepatocytes, chondrocytes, or glial cells, to thereby induce gene transcription. Recently, mice deficient in this gene (knockout mice) have been developed. It was reported that the mice possess weaker defense capability against infections but exhibits alleviated symptoms of inflammation and sepsis as compared with the wild type (Wei, X. et al., (1995) *Nature* **375**, 408-411; Machick, J. D. et al., (1995) *Cell* **81**, 641-650). There is a report showing that iNOS is induced in any species by inflammatory conditions and that the suppression of its enzymatic activities and expression are effective for alleviating the inflammatory symptoms (Moncada, S. and Higgs, E. A. (1995) *FASEB J.* **9**, 1319-1330). Further, in a sepsis model, the administration of iNOS enzyme inhibitors were found to be effective (Kerwin, J. F. et al., (1995) *J. Med. Chem.* **38**, 4343-4362).

The endothelial type NOS (eNOS), on the other hand, was reported to participate in the homeostasis, especially in suppressing the rise in blood pressure, and is considered to play an important role in biological functions. Consequently, it has been desired to find a compound that does not affect the eNOS activity but specifically inhibits iNOS activity. However, since the primary structural domains of the proteins that inhibit the activities of these isozymes resemble each other very closely, none of the present- NOS enzyme inhibitors are satisfactory in terms of their specificities.

On another aspect, it was confirmed that substances that generate NO can suppress vascular hypertrophy, thereby preventing arteriosclerosis and post-angioplasty vascular restenosis in animal models (Garg, U. C. and Hassid, A. J. (1989) *J. Clin. Invest.* **83**, 1774-1777, Cooke, J. P. et al., (1992) *J. Clin. Invest.* **90**, 1168-1172). It was also reported that the forced expression of the NOS gene in VSMC results in an increase of NO production accompanied by the suppression of hypertrophy of the inner membrane of VSMC (von der Leyen, H. E. et al., (1995) *Proc. Natl. Acad. Sci. USA* **92**, 1137-1141). Therefore, it is expected that the generation of NO at the site of vascular hypertrophy can be effective in treating or preventing vascular hypertrophy.

The above-described facts suggest that, if iNOS isozyme-specific gene expression-regulating compounds are found out, the inhibitors will be useful as anti-inflammatory agents, while the inducers will be useful as agents which lead to therapeutic improvements in especially cardiovascular field.

Reporter genes are utilized for monitoring the expression of a certain gene simply and easily with high sensitivity, (Yokota, T., and Arai, K. (1993) *Biomanual Series 4*, Youdosha). Reporter genes are used instead of directly detecting the test gene expression and such genes that are widely being used at present include those whose expression products can be easily assayed, such as chloramphenicol acetyl transferase (CAT), β -galactosidase (β -Gal), and luciferase. The activity of the test DNA, whose regulatory activity is to be measured, can be detected with high sensitivity by inserting it into a plasmid at either upstream or downstream of the reporter gene.

The reporter genes used to examine the regulation of the mouse iNOS (miNOS) gene expression and the results were reported. For example, within the upstream region of the miNOS transcriptional start site (hereinafter referred to as "5'-flanking region"), there was found a region at about 1.7 kb upstream from the transcriptional start site, which is involved in the induction of the miNOS gene in response to lipopolysaccharides (LPS) or IFN- γ . Furthermore, consensus sequence regions, to which transcription factors NF- κ B and IRF-1 are considered to bind, were shown to be essential in the induction of the gene expression (Xie, Q. et al., (1993) *J. Exp. Med.* **177**, 1779-1784, Vodovotz, Y. et al., (1993) *J. Exp. Med.* **178**, 605-613, Martin, E. et al., (1994) *J. Exp. Med.* **180**, 977-984, Xie, Q. et al., (1994) *J. Biol. Chem.* **269**, 4705-4708). According to another report, the region was found within the 5'-flanking region of about 1.6 kb (Lowenstein, C. J. et al., (1993) *Proc. Natl. Acad. Sci. USA* **90**, 9730-9734, Kamijo, R. et al., (1994) *Science* **263**, 1612-1615). In both cases, however, the inducibility was less than 50-fold, which may not be strong enough to reflect the actual highly induc-

ibility of the iNOS gene.

In contrast, it was difficult to prove the existence of cDNA of the human iNOS (hiNOS) gene. One of the reasons for this is that the cells, in which induction was effected by a single cytokine or a combination of two cytokines, were not discovered, unlike the case with the mouse macrophage or the rat VSMC. There is a report that hiNOS cannot be induced in the human macrophage even by a combination of three or more different kinds of cytokines (Weinberg, J. B. et al., (1995) *Blood* **86**, 1184-1195). However, after the report of the first successful cloning of hiNOS cDNA by stimulating human hepatocytes with three or more different kinds of cytokines to induce the gene (Geller, D. A. et al., (1993) *Proc. Natl. Acad. Sci. USA* **90**, 3491-3495), many reports of the hiNOS cDNA cloning emerged, but the regulatory mechanism of the induction has not been elucidated (Sherman, P. A. et al., (1993) *Biochemistry* **32**, 11600-11605, Charles, I. G. et al., (1993) *Proc. Natl. Acad. Sci. USA* **90**, 11419-11423, Hokari, A. et al., (1994) *J. Biochem.* **116**, 575-581). At the same time, the complete structure of the hiNOS structural gene (Figure 1) and its nucleotide sequence of the 5'-flanking region of approximately 0.4 kb were elucidated (Chartrain, N. A. et al., (1994) *J. Biol. Chem.* **269**, 6765-6772).

On the other hand, the present inventors succeeded in cloning the iNOS gene from the rat vascular smooth muscle and reported to suggest that its cDNA is uniform throughout different tissues and species (Nunokawa, Y. et al., (1993) *Biochem. Biophys. Res. Commun.* **191**, 89-94). Furthermore, the present inventors cloned a stretch of over 3.2 kb DNA, which is expected to contain the iNOS gene promoter region, determined the nucleotide sequence of the 5'-flanking region of approximately 1.5 kb, and reported that the sequence contains, as does the miNOS gene sequence, a consensus sequence thought to be regulated by interferon (IFN)- γ and transcription factor NF- κ B (Nunokawa, Y., et al., (1994) *Biochem. Biophys. Res. Commun.* **200**, 802-807).

Thereafter, 16 kb of the 5'-flanking region of the hiNOS gene was studied using a reporter gene. The result showed that a region necessary for the induction of the hiNOS gene exists between 3.8 kb and 16 kb upstream from the transcriptional start site (de Vera, M. E. et al., (1996) *Proc. Natl. Acad. Sci. USA* **93**, 1054-1059). However, the inducibility was still about 10-fold and the expression with no stimulation was quite strong. Thus, this report provides insufficient explanation for the actual and powerful induction of iNOS gene. In addition, the region up to 3.7 kb upstream from the transcriptional start site of the hiNOS gene was shown not to be involved in induction of the gene expression (Laubach, V. E. et al., (1994) *Abstract Book of the 1st International Conference of Biochemistry and Molecular Biology of Nitric Oxide* (UCLA Sunset Village, Los Angeles, CA, USA), A16, Kleinert, H. et al., (1996) *Mol. Pharmacol.* **49**, 15-21).

The present inventors generated cells transfected with a plasmid containing approximately 3.2 kb of the 5'-flanking region of the hiNOS gene, which had been previously cloned, linked to a reporter gene. The results indicated, contrary to those reported by using the miNOS promoter region, but similar to those reported by other groups using the hiNOS gene, that the reporter gene has been expressed even under non-inducing conditions when the 5'-flanking region of approximately 3.2 kb is used, and that the inducibility by cytokines is either absent or very weak (Nunokawa, Y. et al., (1996) *Biochem. Biophys. Res. Commun.* **223**, 347-352). Moreover, the reporter gene expression was detected even in the cells transfected with the above plasmid that do not show the hiNOS induction by various cytokines (Nunokawa, Y. et al., (1996) *Biochem. Biophys. Res. Commun.* **223**, 347-352). That is, it was found that the control of the hiNOS gene expression by inducers such as cytokines cannot be explained based solely on the promoter region which was inferred from the reported results with the miNOS gene. However, the reason for this has not been made clear at all so far.

40 Disclosure of the Invention

The object of the present invention is to provide a method for screening a substance capable of suppressing or inducing the expression of the hiNOS gene by identifying the region(s) of the hiNOS gene necessary for the control of expression by inducing factors, constructing the expression vector containing the region(s), and utilizing the vector for the screening.

As described above, when the upstream region from the hiNOS gene transcriptional start site, i.e., the 5'-flanking region, is inserted upstream of a reporter gene, the reporter gene expression is unexpectedly observed under non-inducing conditions. Based on this finding, we presumed that there was the possibility that regions other than the 5'-flanking region might control the hiNOS gene expression, and carried out experiments.

As a result of examination of the sequence of the hiNOS gene 3'-untranslated region (UTR) (SEQ ID NO:1), the present inventors have found the "AUUUA sequence," which was reported to play a role in destabilizing the mRNA, at four different places within the 3'-UTR of the hiNOS gene (Figure 2, underlined positions). Therefore, the present inventors considered that a certain element, which destabilizes the transcription products of the gene, might control the induction of the hiNOS gene expression.

Many of the reporter genes commonly used today are constructed by incorporating an SV40-derived region having the poly A addition in order to express the mature mRNA. The plasmids so far used to confirm the hiNOS gene expression and the "pGL3 basic plasmid" that the present inventors used in the previous reports are constructed by inserting the SV40-derived region having the poly A addition (SEQ ID NO:2) at the downstream of the reporter gene (Figure 3).

If the induction of the test gene does not entirely depend on the activation of transcription but also is caused by instability of the produced mRNA, it is thus necessary to incorporate the region involved in the instability of the produced mRNA into the expression vector containing the reporter gene.

Since it was considered that the high inducibility, which had to be inherently observed, could not be realized by introducing the reporter system containing only the 5'-flanking region including the promoter, the present inventors additionally inserted a gene fragment of approximately 1 kb containing the hiNOS gene 3'-UTR and 3'-flanking region at downstream of the reporter gene. Consequently, it has been found that these regions function cooperatively with the promoter region to bring about a strong induction. That is, by inserting these regions, it has been found that the reporter gene expression is eliminated under non-inducing conditions and is activated only when cytokines are allowed to act. These results revealed the existence of the regions necessary for the induction of hiNOS gene by cytokines, which had been unknown.

Furthermore, the present inventors constructed a plasmid mimicking the genomic structure of the hiNOS gene except that the open reading frame (ORF) of the hiNOS gene was replaced by the ORF of the reporter gene. Specifically, the hiNOS gene promoter region was inserted upstream and the gene fragment of approximately 1 kb containing the hiNOS gene 3'-UTR and 3'-flanking region was inserted downstream of the reporter gene. By utilizing the human cell line transformed with the plasmid, the present inventors developed a method to screen compounds controlling the hiNOS gene expression rapidly with high sensitivity, thereby completing the present invention.

More specifically, the present invention includes each invention described in claims of this specification.

The DNAs, which constitute the hiNOS gene 5'-flanking region, 5'-UTR, 3'-UTR, and 3'-flanking region used in the present invention, include not only those isolated from human cells but also those produced synthetically. Moreover, these DNAs with chemical modifications, or whose bases are altered by substitution, deletion, or addition, can also be used as long as their ability to control the expression is retained. The DNA constituting the hiNOS gene 3'-flanking region used in the present invention is preferably the one having the sequence described in SEQ ID NO:16 (the sequence outside the enclosure in Figure 7).

A gene encoding any peptide or protein can be used as the reporter gene of the present invention as long as one skilled in the art can measure the activity or the amount produced of its expression product (including the quantity of the mRNA produced). For example, chloramphenicol acetyl transferase (CAT), β -galactosidase (β -Gal), and luciferase can be used by measuring their enzymatic activities. Also, the secreted growth hormone or the like can be utilized by measuring its amount produced by such means as immunological antibody reaction.

The vector in the present invention containing the hiNOS gene expression control sequence can be obtained by inserting the expression control sequence into a replicable vector. Examples of the replicable vector include pUC18 and pGEM-3Z, which are known to be replicable in *E. coli*.

When screening substances capable of influencing the hiNOS gene expression according to the present invention, one can use the cells transformed with the vector containing the hiNOS gene expression control sequences of the present invention, or the naturally occurring cells having the expression control sequences described in the present invention and capable of controlling the hiNOS gene expression. Mammalian cells are preferably used as such cells. Transformation can be effected according to the conventional method. The transformed cells of the present invention can be either those with the vector integrated permanently into the host chromosomes or those with the vector transiently incorporated into the host. The cells with the vector permanently integrated into the host chromosomes can be selected by transfecting the host cells with the vector to be introduced containing a selection marker gene or with the vector to be introduced together with another vector containing a selection marker, and culturing the cells in a medium that allows only those cells having incorporated the selection marker to survive.

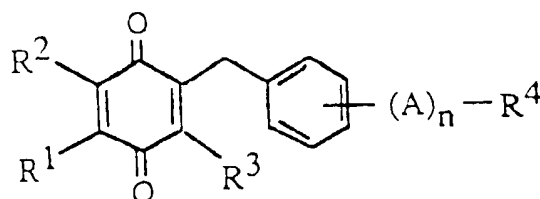
The substances capable of influencing the hiNOS gene expression according to the present invention can be screened, for example, by applying an arbitrary amount of the test substance to the transformed cells that have been cultured for a specified period of time and measuring the amount of the reporter gene expression product after a specified period of time as the enzyme activity or the amount of protein expressed. The test substance may be natural or synthetic. It may also be a single substance or a mixture of substances. For example, it is possible to test a candidate single substance independently, or a mixture of several candidate substances. In addition, it is also possible to test combinatorial libraries. Furthermore, fractions of a mixture such as cell extract can also be tested and repeatedly fractionated to ultimately isolate the substance responsible for influencing the hiNOS gene expression.

On the other hand, according to the present invention, substances capable of altering the activity of a particular substance that influences the hiNOS gene expression can be screened, for example, by applying an arbitrary amount of the candidate substance to the transformed cells that have been cultured for a specified period of time and measuring the changes in the amount of the reporter gene expression product after a specified period of time as the changes in the enzyme activity or in the amount of protein expressed. The candidate substances to be screened are defined as described above for the substances that influence the hiNOS gene expression.

The efficacy of a substance of the present invention capable of suppressing the hiNOS expression or a compound of the present invention capable of suppressing the activity of the compound that induces the hiNOS gene expression

can be measured by determining the reduction in NO excretion into the culture medium, blood, or urine, upon the addition or application of the substance under the conditions that allow the cells capable of expressing mammalian iNOS or their living tissues to produce NO. The amount of NO produced can be measured by the generally known methods represented by the Griess method (Green, L. C. et al., (1982) *Anal. Biochem.* 126, 131-138) described in Example 5-5).

The compounds obtained through the screening method or the screening kit of the present invention as described above are those capable of influencing the amount of the hiNOS gene expressed, for example, those capable of suppressing the hiNOS gene expression, or those capable of altering the activity of a particular compound that influences the amount of the hiNOS gene expressed, for example, those capable of suppressing the activity of a compound that induces the hiNOS gene expression. A specific example of the compound capable of suppressing the activity of a compound that induces the hiNOS gene expression is the compound represented by the following structural formula,



wherein R¹, R², and R³ each independently represent a hydrogen atom, a methyl group, or a methoxy group; A represents an ethylene group or a vinylene group; n represents 0 or 1; and R⁴ represents a hydrogen atom, a hydroxymethyl group, or a carboxyl group which may be esterified or amidated. More specifically, such compounds include the compound obtained in Example 7 of the present specification and represented by the chemical formula as shown in Figure 17 (3-[4-(3,4-dimethoxy-6-methyl-2,5-benzoquinonylmethyl)phenyl]-1-thiomorpholino-1-oxopropane), ethyl 3-[3-(2,5-benzoquinonylmethyl)-phenyl]acrylate, 3-(2,5-benzoquinonylmethyl)benzyl alcohol, 3-(2,5-benzoquinonylmethyl)benzoic acid, 3-[3-(2,5-benzoquinonylmethyl)phenyl]propionic acid, 3-[3-(2,5-benzoquinonylmethyl)-phenyl]propyl alcohol, 3-[3-(2,5-benzoquinonylmethyl)phenyl]-acrylic acid, 3-[4-(3,4-dimethoxy-6-methyl-2,5-benzoquinonylmethyl)phenyl]propionic acid, 2,3-dimethoxy-5-benzyl-6-methyl-1,4-benzoquinone, 3-[4-(3,4-dimethoxy-6-methyl-2,5-benzoquinonylmethyl)phenyl]propanol, and ethyl 3-[3-(3,4-dimethoxy-6-methyl-2,5-benzoquinonylmethyl)phenyl]propionate.

Since the compounds of the present invention can either directly or indirectly suppress the hiNOS gene expression, they are useful as pharmaceutical compositions for treating the pathological conditions or diseases associated with the hiNOS expression abnormality, preferably the pathological conditions or diseases accompanying an excessive expression of the hiNOS gene. For example, the compounds are useful as drugs for treating cardiac and cerebrovascular disorders, ischemic heart disease, septic shock, acute pain, rheumatism, arthritis, asthma, immunodeficiency, viral or non-viral infections, autoimmune diseases, dementia, and cancer (Cattell, V. and Jensen, A., *Histochem. J.* Vol. 27, p777-784, 1995; Ogden, J. E. and Moore, p. K., *TIBTECH* Vol. 13, p70-78, 1995).

When the compounds of the present invention are used as pharmaceutical compositions, they can be administered either orally in the dosage form such as tablets, capsules, elixirs, microcapsules, or parenterally as injections in the form of solutions in water or in other pharmaceutically acceptable liquids, or suspensions. For example, the compounds can be formulated into the above dosage forms by mixing them with physiologically acceptable carriers, flavors, excipients, stabilizers, etc., in a generally accepted form. Additives that can be mixed in tablets include binders such as gelatin, swelling agents such as cornstarch, excipients such as crystalline cellulose, and lubricants such as magnesium stearate. In the case of capsules, liquid carriers can be contained in addition to the components described above. Sterile compositions for injection can also be formulated in the conventional manner.

An aqueous solution for injection is exemplified by an isotonic solution containing glucose, which may also be combined with appropriate solubilizers such as polyethylene glycol. It may further contain buffers, stabilizers, preservatives, antioxidants, and soothing agents. The pharmaceutical preparations thus produced can be administered, for example, to humans and other mammals. Though the dose may vary depending on the symptoms and the like, the preparation is generally administered to an adult about 0.01 mg to about 100 mg, preferably about 0.1 mg to about 50 mg, more preferably - about 1.0 mg to about 25 mg per day in the case of oral administration. In the case of parenteral administration, for example, in the form of injection, an adult is generally intravenously given about 0.001 mg to about 50 mg, preferably about 0.01 mg to about 25 mg, more preferably about 0.1 mg to about 10 mg per day.

Brief Description of the Drawings

Figure 1 shows the entire structure of the hiNOS structural gene.

Figure 2 shows the nucleotide sequence of the 3'-untranslated region (UTR) within the exon 26 of the hiNOS gene. The numerals on the left side represent the nucleotide numbers regarding the first base of the translational start site as 1. The ATTTA sequence is underlined, while the expected poly A addition site is indicated by "↑". The bold-face letters indicate the stop codon in the hiNOS gene. The nucleotide sequences on which the primers were based are marked with their designations and arrows.

Figure 3 shows the structure of the pGL3 basic plasmid and the nucleotide sequence of the SV40-derived poly A addition region. The "Poly A signal" and the "G/T cluster" associated with the poly A addition are underlined.

Figure 4 shows the nucleotide sequences of the primers used for the construction of the plasmids.

Figure 5 is the photograph of a Southern blot of the restriction enzyme digestion fragments (digested with EcoRI, HindIII, and XhoI) of the purified phage genome containing the hiNOS gene 5'-flanking region.

Figure 6 shows the nucleotide sequence substitution to create an NcoI site into the DNA of the hiNOS gene 5'-flanking region. The bold-faced base "T" was substituted with "CA" to construct the mutant plasmid.

Figure 7 shows the nucleotide sequences of the 3'-untranslated region (UTR) and the 3'-flanking region of the hiNOS gene. The numerals on the left side represent the nucleotide numbers regarding the first base of the translational start site as 1. The encircled portion is the 3'-UTR of exon 26. The consensus sequence for the poly A addition (YGT-GTTY) within the 3'-flanking region is underlined. The boldface letters indicate the stop codon in the hiNOS gene. The nucleotide sequences on which the primers were based are marked with their designations and arrows.

Figure 8 shows the structures of expression vectors containing the non-structural regions of the hiNOS gene.

Figure 9 shows the response of the A549 cells transiently transfected with the expression vectors against the cytokine stimulation.

Figure 10 is the photograph of a gel shift assay using the nuclear extracts from the A549 cells stimulated with cytokines.

Figure 11 shows the response of the A 549 cells stably transfected with the expression vector (hiNOSLuc) against the cytokine stimulation.

Figure 12 shows the response of the A 549 cells against the cytokine stimulation, measured by Griess method.

Figure 13 shows the effects of LPS, IL-6, cAMP, and TPA on the response of the A 549 cells stably transfected with the expression vector (hiNOSLuc) against the cytokine stimulation.

Figure 14 shows the effects of the protein kinase inhibitor, staurosporine, on the induced gene expression by cytokines.

Figure 15 shows the effects of dexamethasone on the induced gene expression by cytokines.

Figure 16 shows the effects of the protease inhibitor, TLCK, on the induced gene expression by cytokines.

Figure 17 shows the structural formula of Compound I, which was discovered using the screening system of the present invention.

Figure 18 shows the effects of Compound I on the screening system of the present invention.

Figure 19 is the photograph showing that Compound I suppresses the hiNOS expression at the mRNA level.

Best Mode for Carrying out the Invention

The following Example illustrates the present invention in more detail, but are not to be construed to limit the scope of the present invention.

[Example 1] Construction of expression vectors

1) Cloning of the hiNOS 5'-flanking region

A probe to be used in screening of clones containing the hiNOS 5'-flanking region DNA was prepared by plaque hybridization from a human genome library consisting of 2.5×10^6 phages (Clontech, USA; a phage library incorporated in the EMBL3 vector).

The probe used was the cDNA amplified by polymerase chain reaction (PCR) using as the template: the rat-derived iNOS cDNA (VSM-NOS), which had been previously isolated by the present inventors (Nunokawa, Y. et al., (1993) Biochem. Biophys. Res. Commun. 191, 89-94) and using the SU802 primer (corresponding to bases -138 to -117 of the rat VSM-NOS cDNA/SEQ ID NO:3, Figure 4) and the M1103 primer (corresponding to bases 168 to 188 of the rat VSM-NOS cDNA/SEQ ID NO: 4, Figure 4). PCR was performed using the Taq DNA polymerase (Takara Shuzo) and a buffer accompanied with it. This cDNA obviously possesses high homology to the nucleotide sequence of the 5' end of the hiNOS structural gene. The plaque hybridization was carried out using the ECL direct DNA labeling detection system (Amersham, U.K.) in accordance with the attached experimental protocol.

Positive plaques were purified using the Qiagen Lambda kit (Qiagen, Germany) and digested with EcoRI. Southern blot analysis revealed the DNA fragment of about 5 kb hybridized with the above probe (Figure 5).

The EcoRI-digested fragment of about 5 kb was subcloned into the EcoRI site in the pUC118 plasmid (Takara Shuzo) and a mutant plasmid, in which a base was substituted as shown in Figure 6 (SEQ ID NO:13), was prepared in order to create an NcoI site. This procedure was carried out using Clontech's Site-Directed Mutagenesis kit in accordance with the attached experimental protocol. The mutant plasmid thus prepared was digested with KpnI and NcoI, and the resulting DNA fragment was used in the experiment of 3) below.

2) Cloning of the hiNOS 3'-UTR and a gene fragment about 1 kb downstream therefrom containing the region

The human genome library used in 1) was divided into 60 sub-pools (about 40,000 clones per pool), and the pool containing the 3'-UTR was identified by PCR using the two kinds each of forward primers (SA101/SEQ ID NO:5, SA102/SEQ ID NO:7) and reverse primers (KI101/SEQ ID NO:6, KI102/SEQ ID NO:8), which were designed based on the hiNOS cDNA 3'-UTR sequence (the 3' side of exon 26 in Figure 1 and inside the encircled portion of Figure 7). The positive pool was further subdivided into 30 sub-pools and the pool containing positive clones was identified by the PCR in the same manner as above. At this point, it was estimated that a pool contained not less than 1,000 clones. The phage population containing all the clones in the identified positive sub-pool was cultivated and phage DNA was purified using the Qiagen Lambda kit. From the result that the DNA was amplified the PCR with SA101 and KI101, the clones were confirmed to contain the hiNOS 3'-UTR. The confirmation of the results of PCR was done in the same manner as in 1).

Then, the downstream region containing the 3'-UTR was amplified by PCR using the purified DNA as a template and a forward primer (SA101) and a reverse primer (KI103/SEQ ID NO:9, Figure 4) which was designed based on the sequence of the SP6 promoter portion of the EMBL3 right arm of the phage vector. The sequence of the amplified DNA was determined with the Dye Deoxy terminator kit (Applied Biosystems, USA), through the direct sequencing method using the Applied Biosystems A373 DNA sequencer. The results are shown as SEQ ID NO:14 and Figure 7.

It was confirmed that the amplified DNA is a region containing the hiNOS 3'-UTR. Based on this information, the poly A addition site based on this information was determined and a reverse primer containing an end of the 3'-UTR (KI104/SEQ ID NO:11, Figure 4) was prepared. The KI105 reverse primer (SEQ ID NO:12, Figures 4 and 7) was also prepared based on the sequence of the 3'-flanking region. Using the purified DNA as a template, and SA103 (SEQ ID NO:10) and KI104, the 3'-UTR DNA of approximately 0.5 kb was amplified by PCR. Since SA103 contains an NheI recognition site on its 5' end, and KI104 contains a SalI recognition site on its 5' end, the above DNA was digested with SalI and NheI and the resulting DNA fragment (3'-UTR) was used in the experiments in 3) below.

Using the purified DNA as a template, and SA103 and KI105, the DNA of approximately 1 kb containing the 3'-UTR and the 3'-flanking region was amplified by PCR. Since SA103 contains an NheI recognition site on its 5' end and KI105 contains a BamHI recognition site on its 5' end, the above DNA fragment was digested with BamHI and NheI and the resulting fragment (containing the 3'-UTR and the 3'-flanking region) was used in the experiments in 3) below.

3) Construction of expression vectors containing the non-structural regions of the hiNOS gene

(3-A) Construction of the plasmid (pGLNOS5+SV3) having the 5'-flanking region and the 5'-UTR inserted upstream of the luciferase gene of the pGL3 basic plasmid

The pGL3 basic plasmid (Promega, USA) was digested with restriction enzymes KpnI and NcoI and the DNA fragment prepared in 1) above was inserted therein (Figure 8A).

(3-B) Construction of the plasmid (pGLNOS3A) in which the region stretching from immediately downstream of the luciferase stop codon up to the SV40-derived poly A addition signal region of the pGL3 basic plasmid was deleted and an approximately 1 kb fragment containing the 3'-UTR and the 3'-flanking region of the hiNOS gene was inserted therefor immediately downstream of the luciferase gene in the pGL3 basic plasmid

The pGL3 basic plasmid was digested with BamHI and XbaI and the DNA fragment prepared in 2) above (3'-flanking region) was inserted therein (Figure 8B).

(3-C) Construction of the plasmid (pGLNOS53A) in which the hiNOS gene 5'-flanking region and 5'-UTR were inserted upstream of the luciferase gene in the pGL3 basic plasmid, the region stretching from immediately downstream of the luciferase stop codon up to the SV40-derived poly A addition signal region of the pGL3 basic plasmid was deleted, and an approximately 1 kb fragment containing the 3'-UTR and the 3'-flanking region of the hiNOS gene was inserted therefor

The pGL3 basic plasmid was digested with BamHI and XbaI and the DNA fragment prepared in 2) above (containing the 3'-UTR and the 3'-flanking region) was inserted therein. The resulting plasmid was digested with restriction enzymes KpnI and NarI and the DNA fragment prepared in 1) above was inserted therein (Figure 8C).

(3-D) Construction of the plasmid (pGLNOS53-SV3) in which the region stretching from immediately downstream of the luciferase stop codon up to the SV40-derived poly A addition signal region of the pGLNOS5+SV3 plasmid prepared in (3-A) above was deleted, and the 3'-UTR of the hiNOS gene was inserted therefor immediately down-

stream of the luciferase gene

The pGL3 basic plasmid was digested with restriction enzymes XbaI and SalI, the DNA fragment (3'-UTR) was inserted therein, and it was digested with KpnI and NarI. The DNA fragment obtained by the digestion of pGLNOS5+SV3 with KpnI and NarI was then inserted into this plasmid (Figure 8D).

(3-E) Construction of the plasmid (pGLNOS53+SV3) in which the 3'-UTR of the hiNOS gene was inserted immediately downstream of the luciferase gene in the pGLNOS5+SV3 plasmid prepared in (3-A) above

The pGLNOS53-SV3 plasmid prepared in (3-D) above was digested with restriction enzyme SalI, the ends were made blunt by the DNA Blunting Kit (Takara Shuzo), and it was digested with NarI. The DNA fragment produced by digesting the pGLNOS5+SV3 plasmid with XbaI, the ends were made blunt using the DNA Blunting Kit, followed by digestion with NarI. The resulting DNA fragment was inserted into the above plasmid (Figure 8E).

[Example 2] Reaction of the A549 cells transiently transfected with the expression vectors against the cytokine stimulation

A human lung cancer-derived cell line, A549, which is known to express hiNOS in response to the cytokine stimulation, was obtained from ATCC (Catalog #CCL185) and cultured in the GIT medium (Wako Pure Chemical Industries, Ltd.) in an atmosphere of 5% CO₂ incubator. The expression vectors shown in Figure 8 were each transfected transiently into the A549 cells using LipofectAMINE (Life Technologies Inc., USA). Six hours after adding either human IL-1 β (1 ng/ml) or human IL-1 β (1 ng/ml) + human IFN- γ (1,000 U/ml) + human TNF- α (500 ng/ml) (this mixture will be sometimes hereinafter referred to as "CM"), the response was examined (Figure 9). The luciferase activity was measured in accordance with the protocol for the Luciferase Assay System (Promega). The ARGUS-50 luminometer (Hamamatsu Photonics) was used for detection. Human IL-1 β was purchased from Genzyme (USA). Human IFN- γ and the human TNF- α were produced at Suntory Institute for Biomedical Research by the conventional method. In order to normalize the assay in this experiment, a β -gal expression vector (pSV- β -gal, Promega) was co-transfected as a control vector and the results were extrapolated.

When pGLNOS5+SV3 was introduced, a high level expression was observed without the stimulation and the strong induction by cytokines was not observed. When pGLNOS53+SV3 was introduced, the result was the same as in the case that pGLNOS5+SV3 was introduced. In addition, when pGLNOS53-SV3 was introduced, no increase of the reporter expression was observed in both cases of no stimulation and stimulation with the cytokine. On the other hand, when pGLNOS53A (the plasmid containing the 3'-UTR and the 3'-flanking region of the hiNOS gene) was used, almost no luciferase expression occurred without the stimulation, whereas high expression was observed upon the addition of IL-1 β and the expression level became much higher by the addition of the three kinds of cytokines. Namely, it was found that the regulation of the hiNOS gene expression requires the presence of both the 3'-UTR and the 3'-flanking region.

[Example 3] Gene sequence possibly involved in the hiNOS inducibility

By means of the gel shift method, it is possible to observe the binding of proteins by cytokine stimulation to the double-stranded DNA (5'-AACTGTACACAAGCTGGGGACACTCCCTTTGGAAA-3'/SEQ ID NO:15) which corresponds to the nucleotides -131 to -97 upstream of the transcriptional start site of the hiNOS gene. The above DNA sequence contains the consensus sequence to which NF- κ B is expected to bind. The gel shift method was performed by modifying the above double-stranded DNA with digoxigenin (DIG) (using the Gel Shift Assay kit, Boehringer Mannheim, Germany), incubating it with the nuclear extracts from A549, and subjecting it to 7.5% polyacrylamide gel electrophoresis (Bio-Rad, USA) at 4°C. The cellular nuclear extracts were obtained from the cells stimulated with IL-1 β (1 ng/ml) or with CM for 4 hours or from the non-stimulated cells using the method of Schreiber et al. (Schreiber, E. et al., (1989) Nucleic Acids Res. 17, 6419). The DNA obtained from the gel following the gel electrophoresis was transferred onto the Zeta-Probe nylon membrane (Bio-Rad) by electroblotting (using the apparatus made by Atto), and the DIG-labeled DNA was detected with a chemiluminescent DIG-recognition antibody.

Figure 10 shows the results. It was found that a protein (A), which exists in the A549 nuclear extract in the absence of stimulation, binds to the above sequence (corresponding to band A of Figure 10) and that another binding protein (B), which exists in the A549 nuclear extract is induced to bind to the sequence by the stimulation with IL-1 β or CM (corresponding to band B of Figure 10). These results revealed that 5'-flanking region of the hiNOS gene contains, as does miNOS, the region to which NF- κ B binds upon cytokine stimulation.

[Example 4] Construction of A549 cells stably transfected with expression vectors (A549/hiNOSLuc) and their response to cytokine stimulation

pGLNOS53A and pSV2neo (Clontech) were co-transfected into A549 cells using LipofectAMINE (Life Technologies Inc., USA) and the cells stably transfected with pGLNOS53A (A549/hiNOSLuc) were selected by adding G418 sulfate

(1 mg/ml, Life Technologies Inc.) to the medium.

Six clones were arbitrarily chosen from plural G418 resistant cells. IL-1 β (1 ng/ml) or CM was added thereto and the response was compared after 24 hours with the non-stimulated clones. As a result, all 6 clones expressed luciferase only when they were stimulated with cytokines. These data indicated that the structure of the plasmid to be introduced is important for the induction of the gene expression, but it is not influenced by the position on the chromosome of the introduced gene.

[Example 5] Comparison of the response of A549/hiNOSLuc to cytokine stimulation and the hiNOS expression pattern

A clone (A5) was chosen from among the G418 resistant cells obtained in Example 4 to examine the induction of luciferase expression by a variety of substances listed below. (No particular reason exists for the choice of A5.) The A5 cells were spread onto the 96-well plate to about 10,000 cells/100 μ l per well and cultured for 24 hours. The following substances were added to each well and, after 6 or 24 hours, the luciferase activity was measured in accordance with the Luciferase Assay System (Promega) protocol. The ARGUS-50 luminometer (Hamamatsu Photonics) was used for detection.

1) IL-1 β

The luciferase expression was observed within at least 6 hours by IL-1 β (1 ng/ml) stimulation. However, the same amount of IL-1 β alone did not change the expression level even after 24 hours of stimulation as well as after 6 hours (Figure 11).

2) IFN- γ

IFN- γ (1,000 U/ml) stimulation did not cause an increase of the expression (Figure 11).

3) TNF- α

TNF- α (500 ng/ml) stimulation did not cause an increase of the expression (Figure 11).

4) Combination of cytokines

When the 3 kinds of cytokines used in 1) through 3) were added together (CM), a weak increase of the expression was observed within 6 hours, while a very strong expression was observed after 24 hours (Figure 11). The level of the expression is estimated to be about 500 times as high as the background (100 to 200 counts), which agrees with the report that hiNOS is strongly induced by CM. Additionally, IL-1 β (1 ng/ml) + IFN- γ (1,000 U/ml) also induced the expression strongly, and the 24-hour culture in the presence of the two cytokines resulted in a stronger increase of the expression than IL-1 β (1 ng/ml) alone (Figure 11). Also even in the absence of IL-1 β , the stimulation by IFN- γ and TNF- α caused induction after 24 hours.

5) Comparison with the results obtained using the Griess method

The Griess method, which utilizes the diazotization reaction, is known as a method of indirectly monitoring the production of NO by the cell (Green, L. C. et al., (1982) Anal. Biochem. 126, 131-138). In the method, Griess reagent, which is a mixture of naphthylethylenediamine (Kanto Chemical, Tokyo) and sulfanilic acid (Nacalai Tesque), is reacted with the NO $_2^-$ ion in the culture medium to determine color development by the multititer plate reader (Molecular Devices) by measuring absorbance at 540 nm. When the accumulated NO in the A549 cell culture 48 hours after the cytokine stimulation was measured by this method, the production of NO stimulated by IL-1 β (1 ng/ml) or CM was observed (Figure 12).

These results conform to the assay results of 1) through 4) where the luciferase expression was measured. Therefore, it was confirmed that the hiNOS-derived NO production can be readily calculated by detecting the luciferase expression. (The sensitivity of the method based on the luciferase expression was more than 100 times as high as that of the Griess method.)

[Example 6] Effects of compounds expected to influence the iNOS expression on the assay system of the present invention

1) Effects of LPS (lipopolysaccharides), IL-6, cAMP, TPA (tetradecanoylphorbol acetate), and protein kinase inhibitors

LPS (Sigma, USA), IL-6 (Genzyme), cAMP (dibutyl form, Sigma), and TPA (Nacalai Tesque) were reported to induce iNOS in experimental systems using mouse or rat cells. The luciferase expression was measured in the same manner as in Example 5 in the presence of these compounds. As a result, these substances hardly influenced not only the control levels but also the activation by cytokines (Figure 13). These results revealed that these substances hardly influence the assay system described in Example 5. On the other hand, the protein kinase inhibitor, staurosporine (Wako Pure Chemical Industries), was found to suppress the induced gene expression by IL-1 β (1 ng/ml) or CM at a concentration of not more than 1 μ M (Figure 14).

2) Effects of glucocorticoid

Dexamethasone (Dex) (Wako Pure Chemical Industries) possesses a powerful antiinflammatory effect. One of its mechanisms is known to be attributed to the suppression of activation of the transcription factor NF- κ B (Ray, A. and Prefontaine, K. E. (1994) *Proc. Natl. Acad. Sci. USA* **91**, 752-756). It was also reported to suppress the iNOS expression (Radomski, M. W. et al., (1990) *Proc. Natl. Acad. Sci. USA* **87**, 10043-10047). The luciferase expression was measured in the same manner as in Example 5 in the presence of dexamethasone, and found that the induced gene expression by IL-1 β (1 ng/ml) or CM was suppressed at a concentration of not more than 10 μ M (Figure 15). Moreover, the suppressive effect disappeared if it was added after 1 hour of cytokine stimulation.

3) Effects of protease inhibitors

Proteases are considered to be involved in the activation of NF- κ B through the degradation of I- κ B (a protein that suppresses the activation of NF- κ B in the cytoplasm; Verma, I. M. et al., (1995) *Genes & Dev.* **9**, 2723-2735). One of the protease inhibitors, TLCK (Nacalai Tesque), is known to inhibit the iNOS induction as well as the NF- κ B activation (Grisicavage, J. M. et al., (1995) *Biochem. Biophys. Res. Commun.* **215**, 721-729). The luciferase expression was measured in the same manner as in Example 5 in the presence of TLCK. It was found that TLCK suppresses the induced gene expression by IL-1 β (1 ng/ml) or CM at a concentration of about 10 μ M (Figure 16).

These experimental results also demonstrated that a system as described in Example 5 can be used to screen compounds that participate in the control of hiNOS expression.

[Example 7] Screening for compounds that influence the hiNOS gene expression

As in Example 5, the A5 cells were spread on the 96-well plate at 10,000 cells/100 μ l per well and cultured for 24 hours. Subsequently, specific compounds, which were unknown as to whether or not they influence the hiNOS gene expression, were added either individually or as mixtures and the cells were cultured for another one hour. Then, IL-1 β or TNF- α was added to the culture medium to measure the luciferase activity 24 hours after the addition of IL-1 β or TNF- α in the same manner as in Example 6. As a result of screening numerous compounds and mixtures of compounds, it became clear that Compound I shown in Figure 17 can suppresses the luciferase activity concentration-dependently (Figure 18).

Furthermore, it was confirmed that Compound I (20 μ g/ml), which was found by this screening method to suppress the hiNOS gene expression, suppresses the expression of the hiNOS mRNA in the wild-type A549 cells stimulated by CM (Figure 19). On the other hand, Compound I at the same concentration did not influence the β -actin mRNA expression, which is constitutively expressed in the wild-type A549 cells (Figure 19).

It is also possible to screen compounds that induce the hiNOS gene expression by a similar method to that described in Example 7, for example, by adding test compounds to A5 cells and selecting the compounds which increase the luciferase activity.

Thus, it was demonstrated that it is possible to screen compounds which influence the hiNOS gene expression by means of the screening system of the present invention.

Industrial Applicability

The present invention provides a method of simply and easily screening a compound capable of controlling the expression of human inducible nitric oxide (NO) synthase (hiNOS) with high sensitivity. The method of the present invention also enable convenient and sensitive screening of a compound that is considered to be useful for treating

EP 0 864 648 A1

inflammations and sepsis by suppressing the hiNOS expression, or a compound that is considered to be useful for anti-tumor, antiviral, and vascular restenosis prevention treatments by inducing the expression.

Sequence Listing

5

SEQ ID NO: 1

Sequence length: 604

10

Sequence type: nucleic acid

Strandedness: double

Topology: linear

15

Molecular type: Genomic DNA

Sequence:

	AGCCAGAAGC GCTATCACGA AGATATCTTC GGTGCTGTAT TTCCTTACGA GGCGAAGAAG	60
	GACAGGGTGG CGGTGCAGCC CAGCAGCCTG GAGATGTCAG CGCTCTGAGG GCCTACAGGA	120
20	GGGGTTAAAG CTGCCGGCAC AGAACTTAAG GATGGAGCCA GCTCTGCATT ATCTGAGGTC	180
	ACAGGGCCTG CGGAGATGGA GGAAAGTGAT ATCCCCAGC CTCAAGTCTT ATTCCTCAA	240
	CGTTGCTCCC CATCAAGCCC TTTACTTGAC CTCCTAACAA GTAGCACCCCT GGATTGATCG	300
25	GAGCCTCCTC TCTCAAACCTG GGGCCTCCCT GGTCCCTTGG AGACAAAATC TTAAATGCCA	360
	GGCCTGGCGA GTGGGTGAAA GATGGAACCTT GCTGCTGAGT GCACCACTTC AAGTGACCAC	420
	CAGGAGGTGC TATCGCACCA CTGTGTATTT AACTGCCTTG TGTACAGTTA TTTATGCCTC	480
30	TGTATTTAAA AAACCTAACAC CCAGTCTGTT CCCCATGGCC ACTTGGGTCT TCCCTGTATG	540
	ATTCCTTGAT GGAGATATTT ACATGAATTG CATTTTACTT TAATCACAAA AAAAAAAAAA	600
	AAAA	604

35

SEQ ID NO: 2

Sequence length: 296

Sequence type: nucleic acid

40

Strandedness: double

Topology: linear

Molecular type: Genomic DNA

Sequence:

45	CTAGAGTCGG GGCGGCCGGC CGCTTCGAGC AGACATGATA AGATACATTG ATGAGTTTGG	60
	ACAAACCACA ACTAGAATGC AGTGAAAAAA ATGCTTTATT TGTGAAATTT GTGATGCTAT	120
	TGCTTTATTT GTAACCATT TAAGCTGCAA TAAACAAGTT AACAACAACA ATTGCATTCA	180
50	TTTTATGTTT CAGGTTT CAGG GGGAGGTGTG GGAGGTTTTT TAAAGCAAGT AAAACCTCTA	240
	CAAATGTGGT AAAATCGATA AGGATCCGTC GACCGATGCC CTTGAGAGCC TTCAAC	296

55

SEQ ID NO: 3

EP 0 864 648 A1

Sequence length: 22

Sequence type: nucleic acid

5 Strandedness: single

Topology: linear

Molecular type: other nucleic acid (synthetic DNA)

10 Sequence:

CTTCTCAGCC ACCTTGGTGA GG

22

SEQ ID NO: 4

15 Sequence length: 21

Sequence type: nucleic acid

Strandedness: single

20 Topology: linear

Molecular type: other nucleic acid (synthetic DNA)

TTCTGTGCAG TCCAGTCAG G

21

25 SEQ ID NO: 5

Sequence length: 19

Sequence type: nucleic acid

30 Strandedness: single

Topology: linear

Molecular type: other nucleic acid (synthetic DNA)

35 Sequence:

AGCCAGAAGC GCTATCACG

19

40 SEQ ID NO: 6

Sequence length: 27

Sequence type: nucleic acid

Strandedness: single

45 Topology: linear

Molecular type: other nucleic acid (synthetic DNA)

Sequence:

50 TGTGATTAAA GTAAAATGCA ATTTCATG

27

55 SEQ ID NO: 7

EP 0 864 648 A1

Sequence length: 22

Sequence type: nucleic acid

5 Strandedness: single

Topology: linear

Molecular type: other nucleic acid (synthetic DNA)

10 Sequence:

GCCTGGAGAT GTCAGCGCTC TG

22

15 SEQ ID NO: 8

Sequence length: 21

Sequence type: nucleic acid

Strandedness: single

20 Topology: linear

Molecular type: other nucleic acid (synthetic DNA)

Sequence:

25 GGGGAACAGA CTGGGTGTTA G

21

SEQ ID NO: 9

30 Sequence length: 19

Sequence type: nucleic acid

Strandedness: single

Topology: linear

35 Molecular type: other nucleic acid (synthetic DNA)

Sequence:

CATTAGGTG ACACTATAG

19

40

SEQ ID NO: 10

Sequence length: 29

45 Sequence type: nucleic acid

Strandedness: single

Topology: linear

Molecular type: other nucleic acid (synthetic DNA)

50

Sequence:

GGCGCTAGCC TACAGGAGGG GTTAAAGCT

29

55

EP 0 864 648 A1

SEQ ID NO: 11

Sequence length: 39

Sequence type: nucleic acid

Strandedness: single

Topology: linear

Molecular type: other nucleic acid (synthetic DNA)

Sequence:

GCGGCCGCGT CGACGATTAA AGTAAAATGC AATTCATGT

39

SEQ ID NO: 12

Sequence length: 26

Sequence type: nucleic acid

Strandedness: single

Topology: linear

Molecular type: other nucleic acid (synthetic DNA)

Sequence:

GCGCGGATCC GGCCCACTCT CCTAAG

26

SEQ ID NO: 13

Sequence length: 27

Sequence type: nucleic acid

Strandedness: double

Topology: linear

Molecular type: other nucleic acid (synthetic DNA)

Sequence:

ATGGCCTGTC CCATGGAAAT TTCTGTT

27

SEQ ID NO: 14

Sequence length: 1026

Sequence type: nucleic acid

Strandedness: double

Topology: linear

Molecular type: Genomic DNA

Sequence:

TGAGGGCCTA CAGGAGGGGT TAAAGCTGCC GGCACAGAAC TTAAGGATGG AGCCAGCTCT

60

EP 0 864 648 A1

5 GCATTATCTG AGGTCACAGG GCCTGGGGAG ATGGAGGAAA GTGATATCCC CCAGCCTCAA 120
 GTCTTATTTC CTCAACGTTG CTCCTCATCA AGCCCTTTAC TTGACCTCCT AACAAAGTAGC 180
 ACCCTGGATT GATCGGAGCC TCCCTCTCTCA AACTGGGGCC TCCCTGGTCC CTTGGAGACA 240
 AAATCTTAAA TGCCAGGCCT GGCGAGTGGG TGAAAGATGG AACTTGCTGC TGAGTGCACC 300
 ACTTCAAGTG ACCACGAGGA GGTGCTATCG CACCACTGTG TATTTAACTG CCTTGTGTAC 360
 10 AGTTATTTAT GCCTCTGTAT TAAAAAACT AACACCCAGT CTGTTCCCCA TGGCCACTTG 420
 GGTCTTCCCT GTATGATTCC TTGATGGAGA TATTACATG AATTGCATTT TACTTTAATC 480
 ACACTGTATG CGTGTGTGGG TGTTTTGTAG GGAAAGCTCT TCTCAGAGTG GGGAGCTGGT 540
 GGGTGTCACT GCCTGGACAG ATCCCCGACA GAGGGACACC CCAGCCAGTC CATGGCTCCT 600
 15 CTGAAATGGC TGCCAGGTGT GCCAGCAGCA GATGGAGCTT CGTGCTGGTC CAAAGACCTG 660
 TGGTAGGGCA GGGGGCGCAG GCCTGCCTCC CACACAAAGT ATCTGAAACG GGGTCTGGTG 720
 AGGGTGGGAT TGTGCGATAA GGCCAGTGTG TCGAGGAAGG CCTTGAGCTT CTTCTTGGAC 780
 20 ACTGTCTTAG AAAGCGTTTT GCTCTGGGGC CACCACTCTC ATGCGAGACT GTGTGCCTTG 840
 GCCAGTACGG ATGTGGTCCC TGGGAAGGCA GCGTGTGAG GCGAGTGTGG GCCACAACAT 900
 CCTCGCCTGA GGGACTGGGG ACCCTCTTGG GTTTGGAGCA GGCCAAGGAA TCCTTCTTAG 960
 GAGAGTGGGC CCCGTTTCCT TCTCCTGGTC AGAACCCAAA AAGGAGCTCA GCGGCGGCCA 1020
 25 CTGGGG 1026

30 SEQ ID NO: 15
 Sequence length: 25
 Sequence type: nucleic acid
 Strandedness: double
 Topology: linear
 35 Molecular type: Genomic DNA
 Sequence:
 AACTGTACAC AAGCTGGGGA CACTCCCTTT GGAAA 25

40
 45 SEQ ID NO: 16
 Sequence length: 544
 Sequence type: nucleic acid
 Strandedness: double
 Topology: linear
 Molecular type: Genomic DNA
 50 Sequence:
 ACTGTATGCG TGTGTGGGTG TTTGTAGGG AAAGCTCTTC TCAGAGTGGG GAGCTGGTGG 60

EP 0 864 648 A1

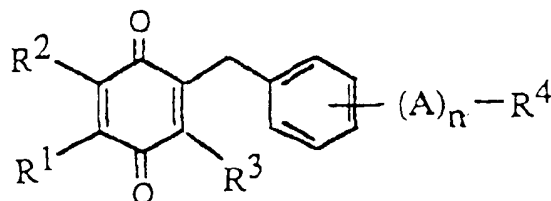
GTGTCACAGC CTGGACAGAT CCCCACAGAG GGGACACCCC AGCCAGTCCA TGGCTCCTCT 120
 GAAATGGCTG CCAGGTGTGC CAGCAGCAGA TGGAGCTTCG TGCTGGTCCA AAGACCTGTG 180
 5 GTAGGGCAGG GGGCGCAGGC CTGCCTCCCA CACAAAGTAT CTGAAACGGG GTCTGGTGAG 240
 GGTGGGATTG TCGCATAAGG CCAGTGTTC GAGGAAGGCC TTGAGCTTCT TCTTGACAC 300
 TGTCTTACAA AGCGTTTTC TCTGGGGCCA CCAGTCTCAT GCGAGACTGT GTGCCTTGGC 360
 10 CAGTACGGAT GTGGTCCCTG GGAAGGCAGC GTGTCGAGGC GAGTGTGGGC CACAACATCC 420
 TCGCCTGAGG GACTGGGGAC CCTCTTGGGT TTGGAGCAGG CCAAGGAATC CTTCTTAGGA 480
 GAGTGGGCCC CGTTTCCTTC TCCTGGTCAG AACCCAAAAA GGAGCTCAGC GGCGGCCACT 540
 15 GGGG 544

20 Claims

1. A DNA containing at least part of the 3'-flanking region of the hiNOS gene.
2. A DNA of Claim 1, wherein said 3'-flanking region of the hiNOS gene has the nucleotide sequence of SEQ ID NO:16.
- 25 3. An expression controlling sequence which comprises:
 - 1) the DNA described in Claim 1 or Claim 2, and
 - 2) at least part of the 3'-UTR of the hiNOS gene.
- 30 4. An expression vector containing the expression controlling sequence of Claim 3.
5. An expression vector of Claim 4, which further contains a reporter gene.
- 35 6. An expression vector which contains the 5'-flanking region including a promoter of the hiNOS gene, a reporter gene, and the expression controlling sequences of Claim 3, in this order from the 5' side.
7. A cell transformed with the expression vector of Claim 5 or Claim 6.
- 40 8. A method of screening a substance capable of influencing the hiNOS gene expression, which comprises treating cells having the expression controlling sequence of Claim 3 with a test substance and observing the changes in the reporter gene expression level.
- 45 9. A screening method of Claim 8, wherein said cells having the expression controlling sequence are the cell of Claim 7.
10. A screening method of Claim 8, wherein said test substance is a mixture.
- 50 11. A kit for screening a substance capable of influencing the hiNOS gene expression, which comprises the cell of Claim 7.
12. A method for screening a substance capable of altering the activity of a specific substance that influences the hiNOS gene expression, which comprises treating cells having the expression controlling sequence of Claim 3 with the specific substance that influences the hiNOS gene expression and a test substance, and observing the changes in the reporter gene expression level.
- 55 13. A screening method of Claim 12, wherein said cells having the expression controlling sequence are the cells of

Claim 7.

14. A screening method of Claim 12, wherein said test substance is a mixture.
- 5 15. A kit for screening a substance capable of altering the activity of a specific substance that influences the hiNOS gene expression, which comprises the cell of Claim 7.
16. A compound capable of influencing the hiNOS gene expression, which is obtainable by the screening method according to any one of Claims 6 to 10.
- 10 17. A compound of Claim 16, which suppresses the hiNOS gene expression.
18. A pharmaceutical composition for treating diseases accompanying abnormal expression of the hiNOS gene, which comprises the compound of Claim 16 as an effective ingredient.
- 15 19. Use of the compound of Claim 16 for preparation of a pharmaceutical composition for treating diseases accompanying abnormal expression of the hiNOS gene.
- 20 20. A method for treating or preventing diseases accompanying abnormal expression of the hiNOS gene, which comprises administering the compound of Claim 16.
21. A pharmaceutical composition for treating diseases accompanying excessive expression of the hiNOS gene, which comprises the compound of Claim 17 as an effective ingredient.
- 25 22. Use of the compound of Claim 17 for preparation of a pharmaceutical composition for treating diseases accompanying excessive expression of the hiNOS gene.
23. A method for treating or preventing diseases accompanying excessive expression of the hiNOS gene, which comprises administering the compound of Claim 17.
- 30 24. A compound capable of altering the activity of a specific substance that influences the hiNOS gene expression, which is obtainable by the screening method according to any one of Claims 12 to 14.
25. A compound of Claim 24, which suppresses the activity of compounds that induce the hiNOS gene expression.
- 35 26. A compound of Claim 25 represented by the following chemical formula:



wherein R^1 , R^2 and R^3 each independently represent a hydrogen atom, a methyl group, or a methoxy group; A represents an ethylene group or a vinylene group; n represents 0 or 1; and R^4 represents a hydrogen atom, a hydroxymethyl group, or a carboxyl group which may be esterified or amidated.

27. A pharmaceutical composition for treating diseases accompanying abnormal expression of the hiNOS gene, which comprises the compound of Claim 24 as an effective ingredient.
- 55 28. Use of the compound of Claim 24 for preparation of a pharmaceutical composition for treating diseases accompanying abnormal expression of the hiNOS gene.
29. A method for treating or preventing diseases accompanying abnormal expression of the hiNOS gene, which com-

EP 0 864 648 A1

prises administering the compound of Claim 24.

30. A pharmaceutical composition for treating diseases accompanying excessive expression of the hiNOS gene, which comprises the compound of Claim 25 or Claim 26 as an effective ingredient.

5

31. Use of the compound of Claim 25 or Claim 26 for preparation of a pharmaceutical composition for treating diseases accompanying excessive expression of the hiNOS gene.

32. A method for treating or preventing diseases accompanying excessive expression of the hiNOS gene, which comprises administering the compound of Claim 25 or Claim 26.

10

15

20

25

30

35

40

45

50

55

Figure 1

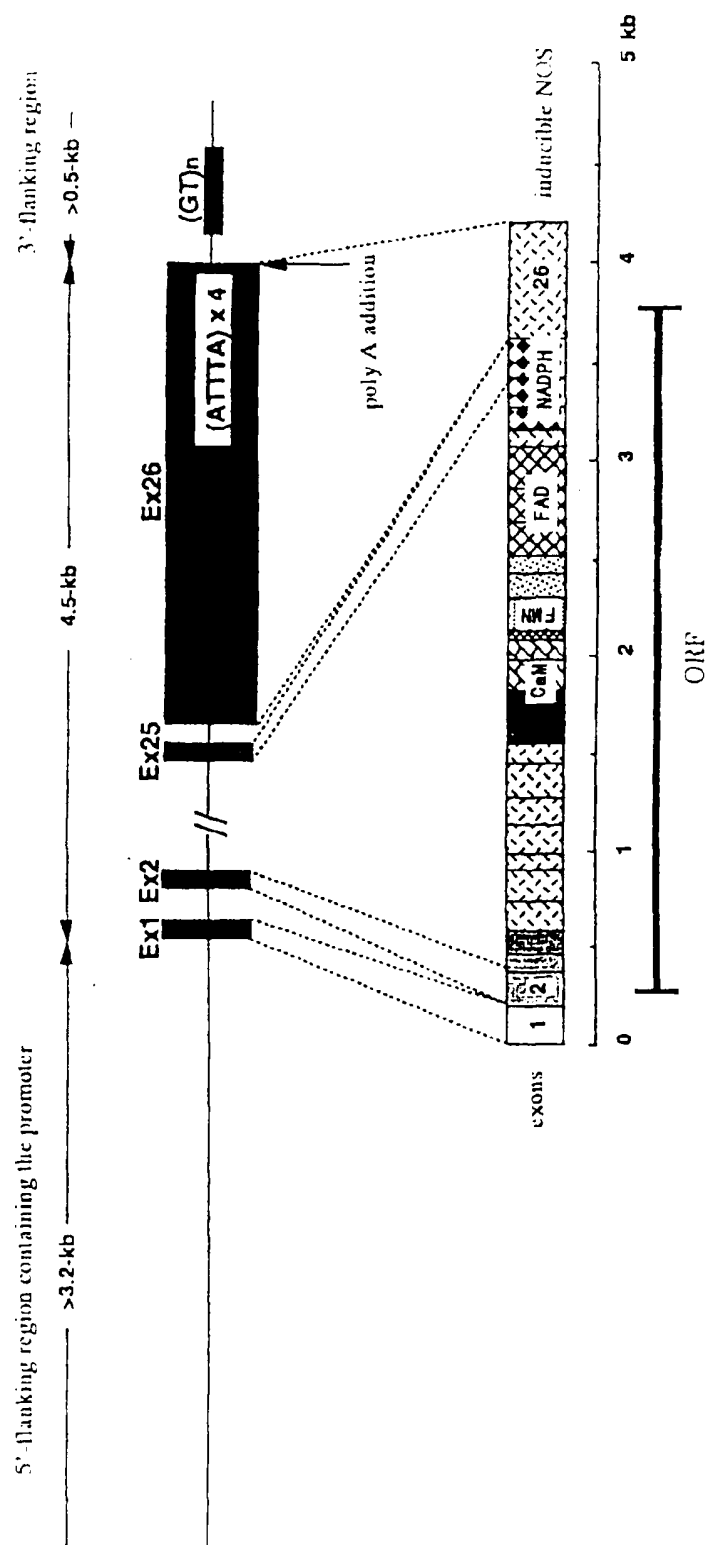


Figure 3

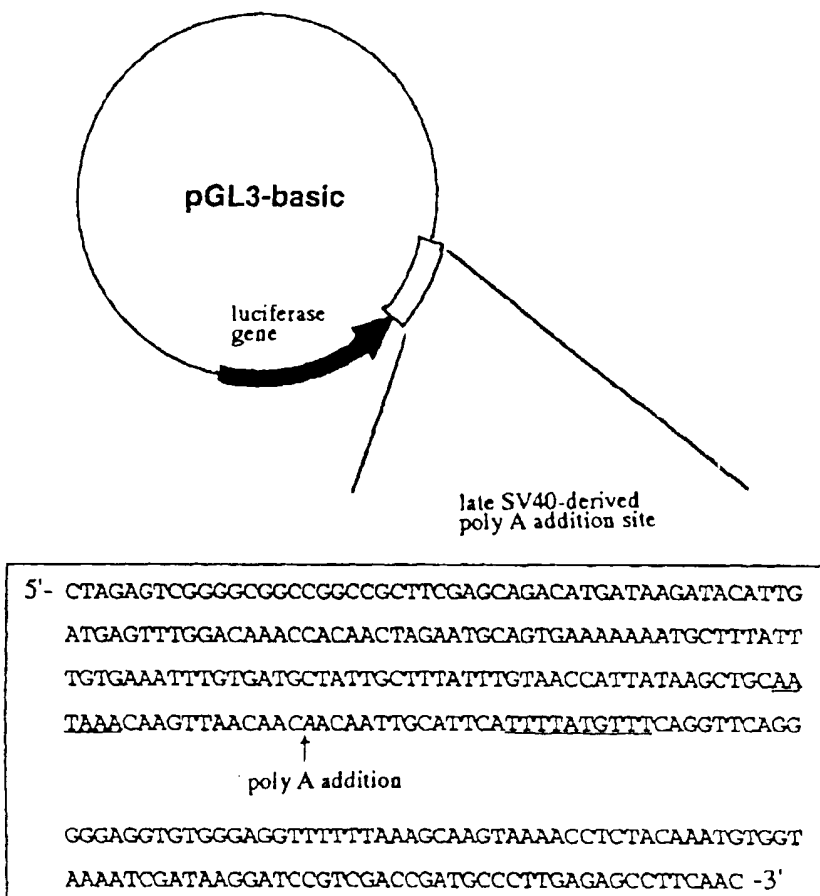


Figure 4

<i>SU802</i>	5'-CTTCTCAGCCACCTTGGTGAGG-3'
<i>MI103</i>	5'-TTCTGTGCAGTCCCAGTGAGG-3'
<i>SA101</i>	5'-AGCCAGAAGCGCTATCACG-3'
<i>KI101</i>	5'-TGTGATTAAAGTAAAATGCAATTCATG-3'
<i>SA102</i>	5'-GCCTGGAGATGTCAGCGCTCTG-3'
<i>KI102</i>	5'-GGGGAACAGACTGGGTGTTAG-3'
<i>KI103</i>	5'-CATTTAGGTGACACTATAG-3'
<i>SA103</i>	5'-GGCGCTAGCCTACAGGAGGGGTAAAGCT-3'
<i>KI104</i>	5'-GCGGCCGCGTCGACGATTAAAGTAAAATGCAATTCATGT-3'
<i>KI105</i>	5'-GCGCGGATCCGGCCCACTCTCCTAAG-3'

Figure 5

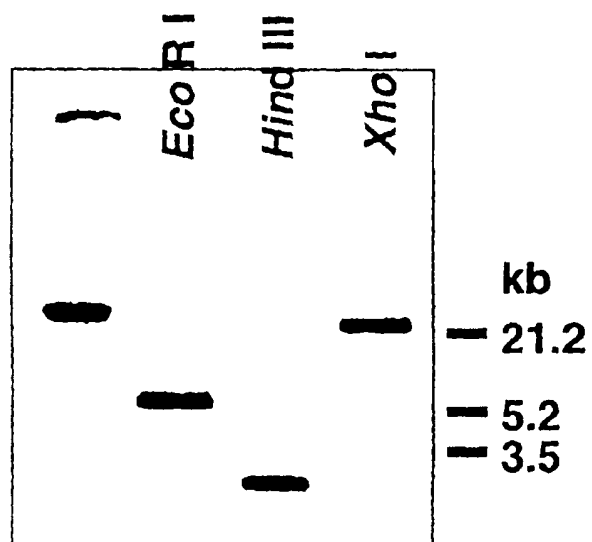


Figure 6

wild-type hiNOS cDNA sequence	5' -ATGGCCTGTCC <u>T</u> TGGAAATTTCTGTT-3'
mutant sequence	5' -ATGGCCTGTCC <u>CA</u> TGGAAATTTCTGTT-3'

Figure 8

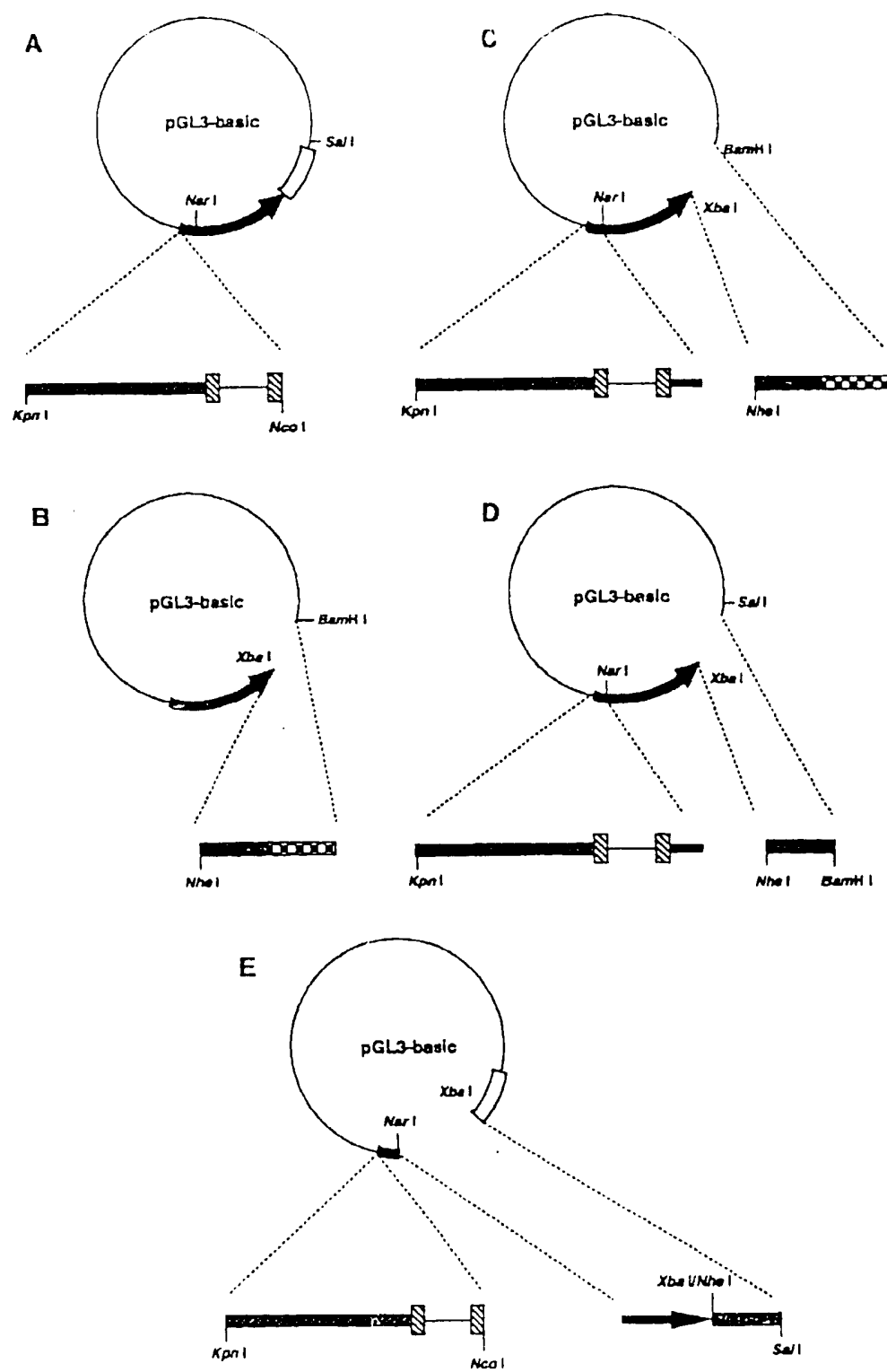


Figure 9

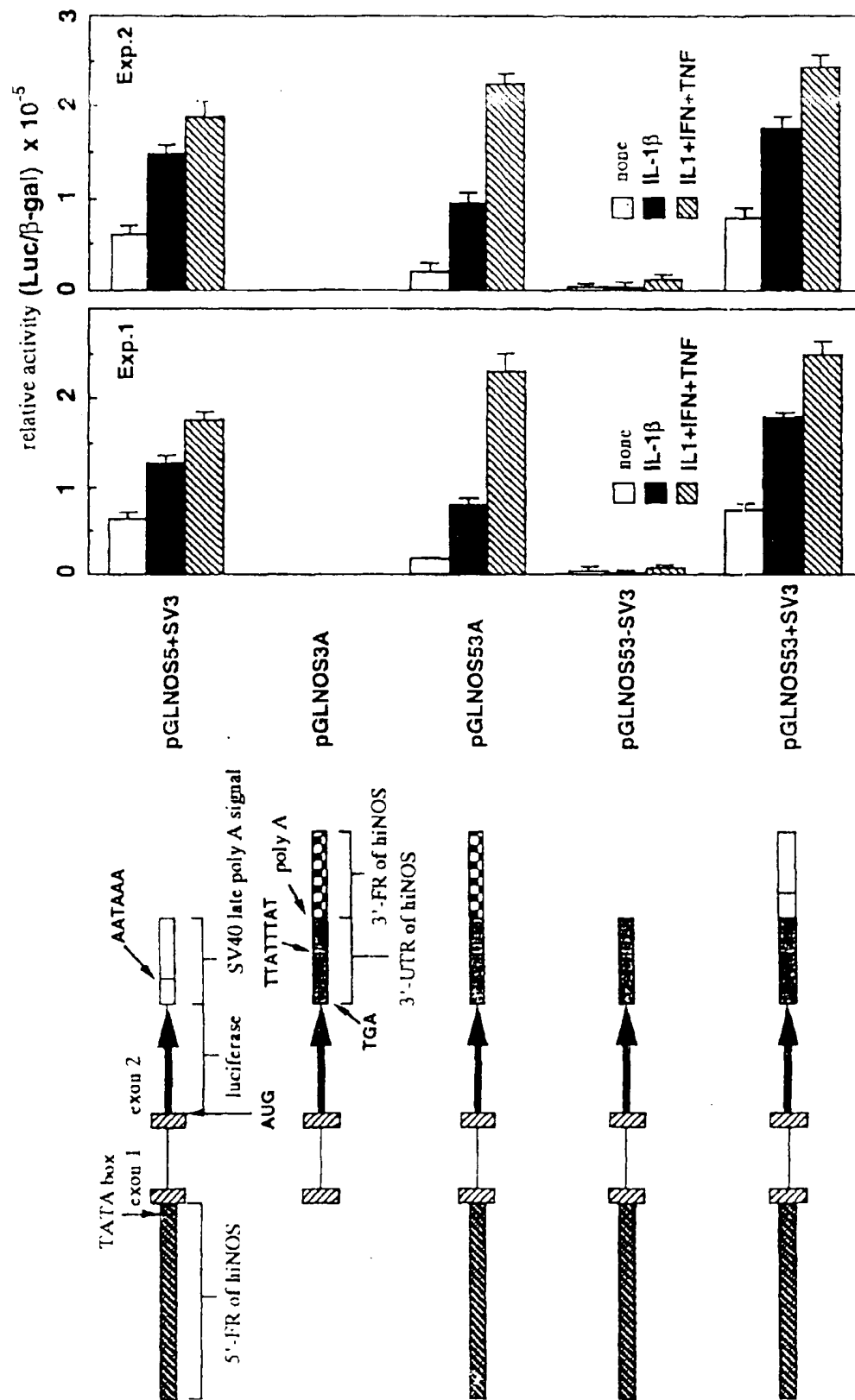
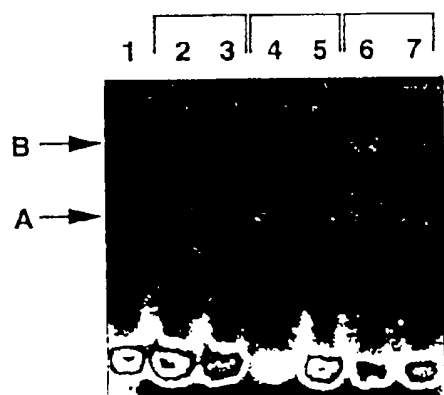


Figure 10



Gel shift assay using the nuclear extract
from A549 cells stimulated by cytokines

- 1: no extract
- 2, 3: no cytokine stimulation
- 4, 5: 3-hr stimulation by IL-1 β
- 6, 7: 3-hr stimulation by CM

A 20-fold concentration of the DIG-free probe was added to 3, 5, and 7.

Figure 11

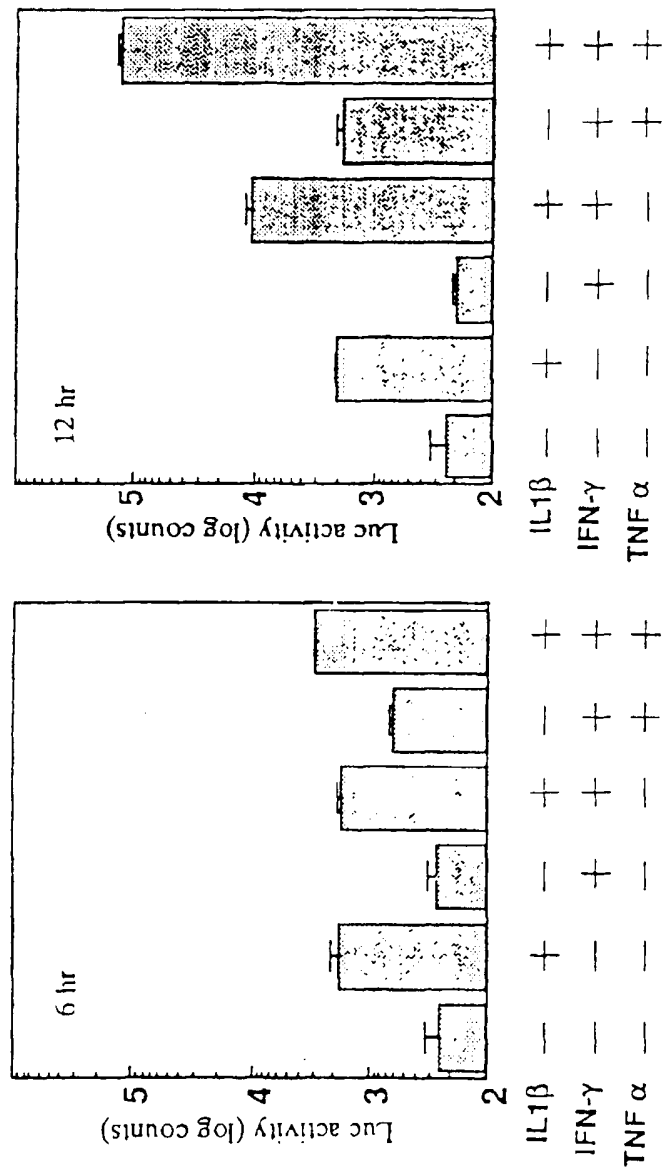


Figure 12

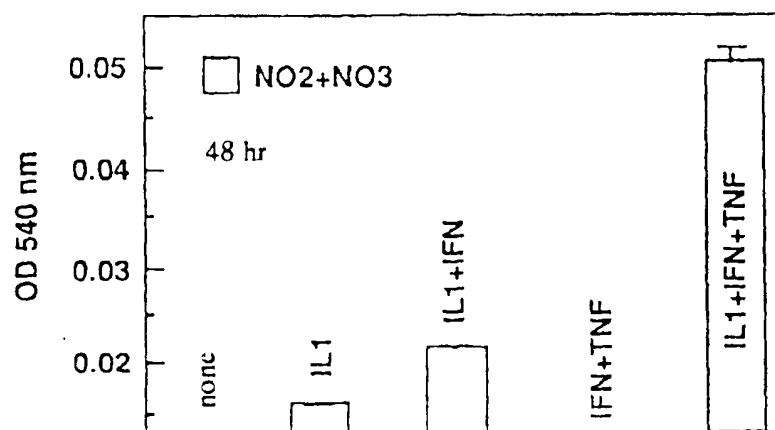
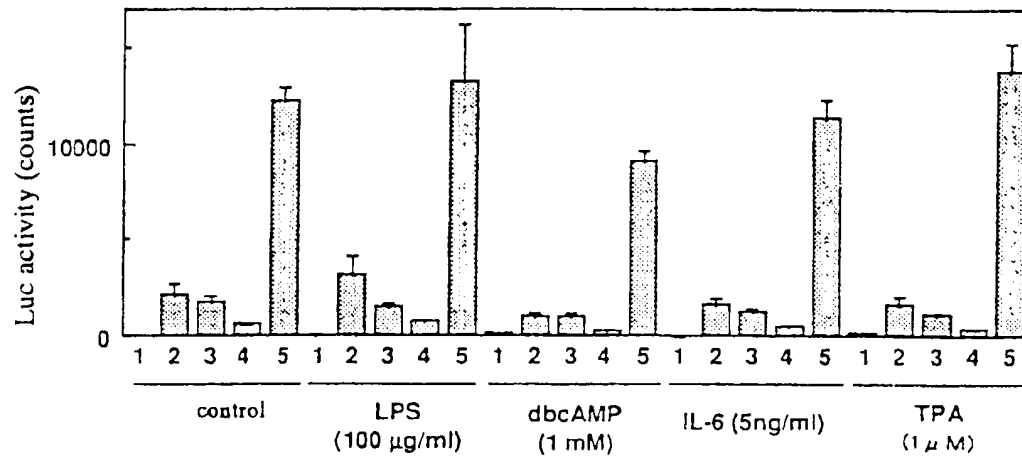


Figure 13



1; control
2; IL-1 β + IFN- γ
3; IL-1 β + TNF- α
4; IFN- γ + TNF- α
5; IL-1 β + IFN- γ + TNF- α

IL-1 β 1 ng/ml
IFN- γ 1000 U/ml
TNF- α 500 ng/ml

Figure 14

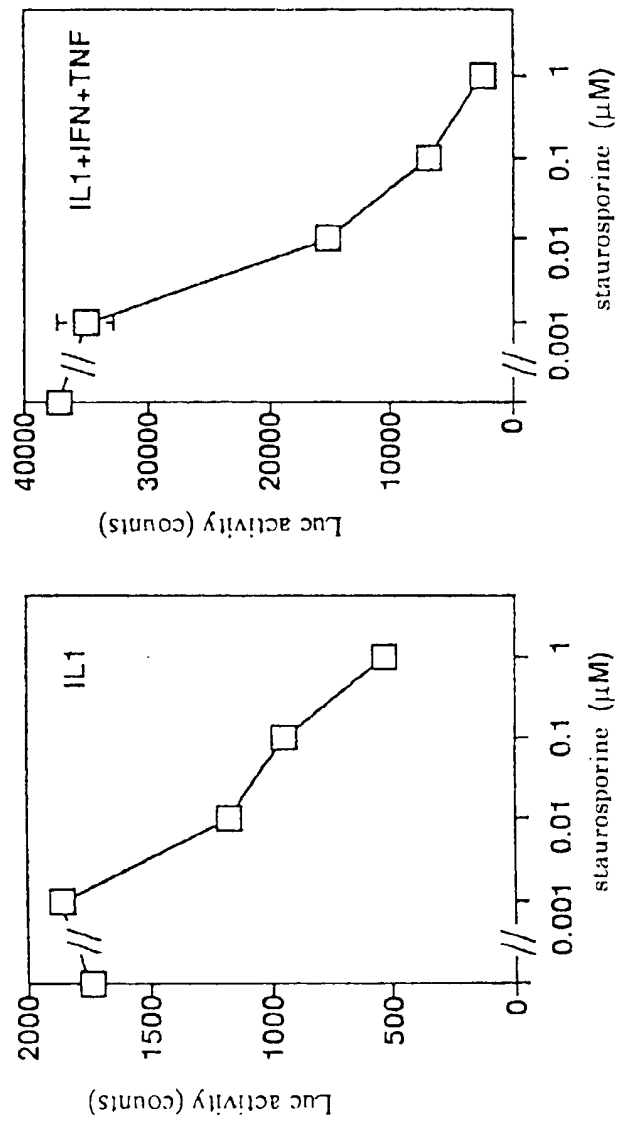


Figure 15

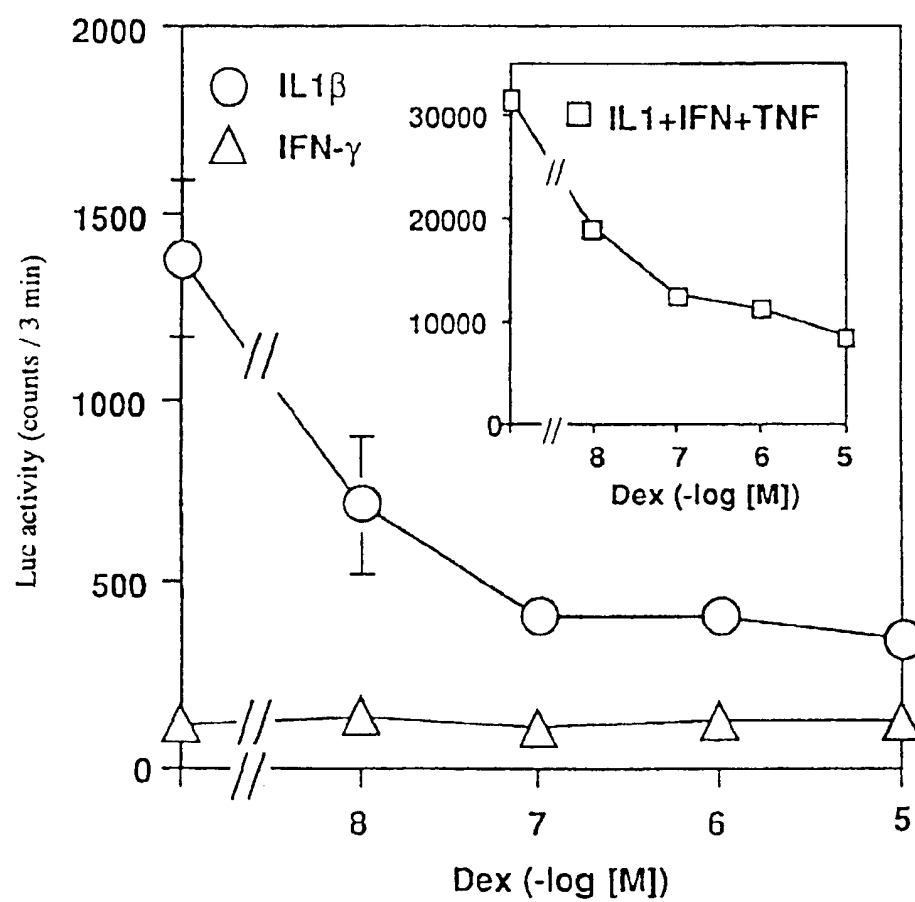


Figure 16

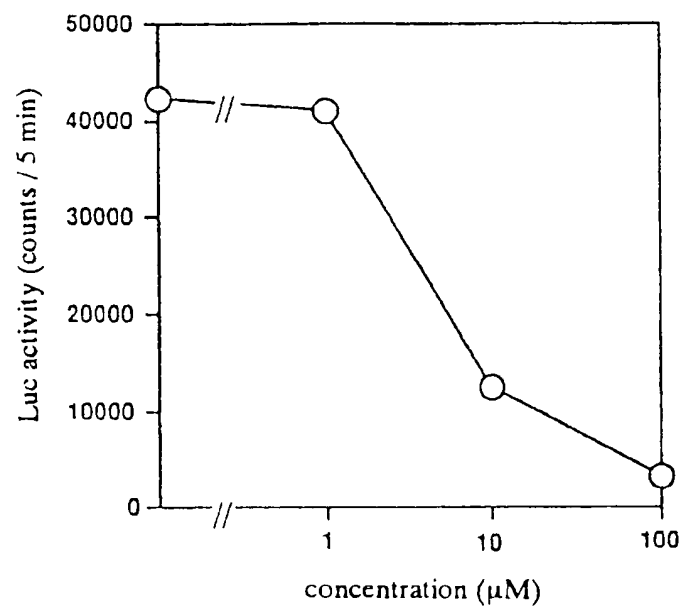


Figure 17

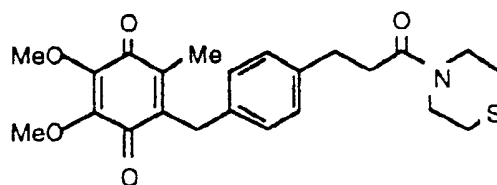


Figure 18

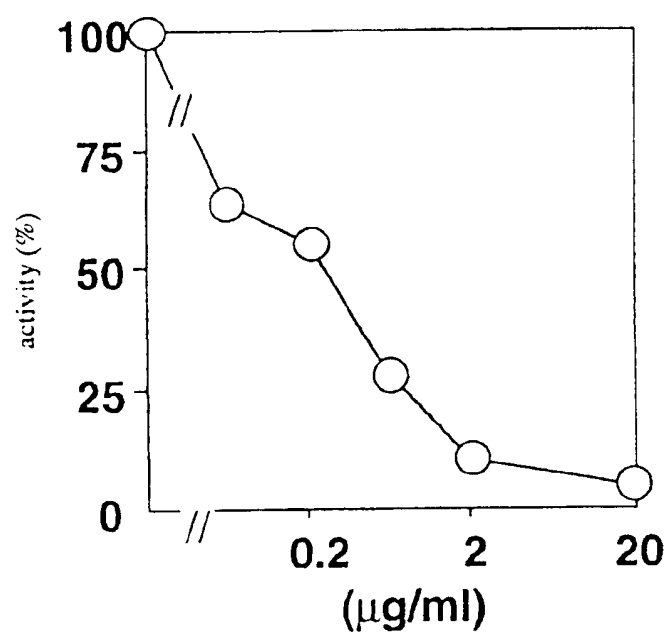
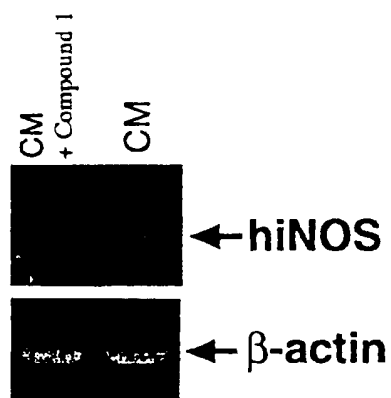


Figure 19



INTERNATIONAL SEARCH REPORT

International application No.

PCT/JP97/03303

A. CLASSIFICATION OF SUBJECT MATTER Int. Cl. ⁶ C12N15/09, C12N15/11, C12Q1/02, C12Q1/25, G01N33/53, C07C50/06, C07C50/30, C07C66/00, C07D295/16, A61K31/12, A61K31/165, A61K31/215 According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) Int. Cl. ⁶ C12N15/09, C12N15/11, C12Q1/02, C12Q1/25, G01N33/53, C07C50/06, C07C50/30, C07C66/00, C07D295/16, A61K31/12, A61K31/165, A61K31/215 Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) WPI (DIALOG), BIOSIS (DIALOG), CA (STN), GenBank/EMBL		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	MICHAEL E. DE VERA et al., "Transcriptional regulation of human inducible nitric oxide synthase (NOS2) gene by cytokines: Initial analysis of the human NOS2 promoter", Proc. Natl. Acad. Sci. USA, Vol. 93 (Feb. 1996) p. 1054-1059	1-19, 21, 22, 24-28, 30, 31
A	NUNOKAWA Y. et al., "PROMOTER ANALYSIS OF HUMAN INDUCIBLE NITRIC OXIDE SYNTHASE GENE ASSOCIATED WITH CARDIOVASCULAR HOMEOSTASIS", BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS, Vol. 200, No. 2 (Apr. 29, 1994) p. 802-807	1-19, 21, 22, 24-28, 30, 31
<input type="checkbox"/> Further documents are listed in the continuation of Box C. <input type="checkbox"/> See patent family annex.		
* Special categories of cited documents: "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family		
Date of the actual completion of the international search December 12, 1997 (12. 12. 97)		Date of mailing of the international search report December 24, 1997 (24. 12. 97)
Name and mailing address of the ISA/ Japanese Patent Office Facsimile No.		Authorized officer Telephone No.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/JP97/03303

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons.

1. ☒ Claims Nos.: 20, 23, 29, 32
because they relate to subject matter not required to be searched by this Authority, namely:
Claims 20, 23, 29 and 32 pertain to methods for treatment of the human or animal body by therapy.
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest ☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.